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Liver-Derived CTL in Hepatitis C Virus Infection: Breadth and Specificity of Responses in a Cohort of Persons with Chronic Infection

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Hepatitis C virus (HCV) is an ssRNA virus that infects the liver. Although parenteral exposure is identified as the transmission risk factor in the majority of individuals, a large proportion of cases has no identifiable risk exposure. Acute infection with HCV leads to chronic infection in at least 80% of exposed individuals, with a subsequent increased risk of cirrhosis, liver failure, and hepatocellular carcinoma. The spontaneous clearance rate of HCV infection is exceedingly low; and IFN-α, the only FDA-approved therapy, leads to viral eradication in less than 10% of cases. Hence, there is interest in immune based therapies to treat established infections. Understanding the immune response to HCV infection will be important to the development of these immune based therapies.

CTL potentially play a major role in the pathogenesis of chronic HCV infection, since they are capable of recognizing virus-infected cells and responding either directly, by lysis of the infected cell, or indirectly, by secreting cytokines that inhibit viral replication and/or recruit other nonspecific inflammatory cells to the liver. Although specific HLA class I-restricted CTL in the liver of chronically infected individuals do not seem to lead to viral eradication, CTL have been shown to play an important role in several viral infections and may play a positive role by controlling the viral load in HCV infection. However, not all studies have shown a correlation of CTL with viral load, and it has been suggested that the chronic inflammatory response can also cause hepatic damage leading to cirrhosis.

We and others have reported previously that HLA class I-restricted CD8+ T cells, which specifically recognize HCV Ags, can be detected among the liver-derived T cells in subjects with chronic HCV infection. We present results from the studies of 44 subjects with chronic HCV infection in whom lymphocytes derived from liver biopsy specimens were expanded polyclonally in Ag-nonspecific manner. The breadth of the CTL responses was assessed using recombinant vaccinia constructs to express the translated polypeptide of two different genotype 1a strains (HCV-1 and HCV-H) on autologous B lymphoblastoid cell lines (B-LCL), such that it was possible to screen for HCV-specific CTL responses presented by all HLA class I alleles expressed in each individual subject. Once CTL responses were detected, the optimum epitope responsible for CTL recognition was determined through the use of synthetic peptides. These results, which build on previous published data, indicate that the HCV-specific CTL response is quite heterogeneous in persons with chronic HCV infection.
Materials and Methods

Subjects

From 1991 to 1995, 44 subjects with evidence of chronic HCV infection (second generation ELISA-positive, persistently elevated serum alanine aminotransferase levels of more than four times the upper limits of normal for at least 6 mo) were evaluated by liver biopsy, using a modified Klatskin technique, as routine assessment for consideration of IFN therapy. All subjects were HBV and HIV-1 seronegative and had hepatic histologic features consistent with the diagnosis of chronic HCV infection. Peripheral blood was also obtained at the time of liver biopsy. Sera or plasma were stored as 1-mL aliquots at ~80°C. PBMC were isolated by Ficoll-Hypaque density centrifugation for evaluation of CTL activity and establishment of autologous B-LCL through EBV transformation. HLA typing was performed on additional samples of peripheral blood by Massachusetts General Hospital Tissue Typing Laboratory (Boston, MA), using standard serologic techniques. Informed consent was obtained from all subjects, and the study was approved by Massachusetts General Hospital and Boston City Hospital Human Studies Committees (Boston, MA). Subjects were coded by the year of receipt of the liver specimen in the laboratory and lettered sequentially.

Stimulation of liver-infiltrating lymphocytes

CD8+ liver-infiltrating lymphocytes were expanded from the liver biopsy specimens, as described (18), using the bispecific mAb CD3,4b (21) at 0.5 × 106 cells/ml of irradiated (30 Gy) allogeneic PBMC in RPMI 1640 media. Peptides were 20 aa in length, overlapping adjacent peptides by 10 aa. Fine mapping was achieved using additional smaller peptides in free acid form that were synthesized on an automated peptide synthesizer (model 432A; Applied Biosystems, Foster City, CA). All peptides were reconstituted in sterile distilled water containing 10% DMSO (Sigma Chemical Co.) and 1 mM DTT (Sigma Chemical Co.).

Synthetic peptides

Peptides corresponding to the aa sequences of the HCV-1 strain were synthesized as free acids by Cambridge Research Biochemicals (Cambridge, MA) or Chiron Mimotopes (Victoria, Australia) using the Fmoc method. Peptides were 20 aa in length, overlapping adjacent peptides by 10 aa. For aa 906–1619, the overlapping peptides were 13 aa in length and overlapped by 10 aa. Fine mapping was achieved using additional smaller peptides in free acid form that were synthesized on an automated peptide synthesizer (model 432A; Applied Biosystems, Foster City, CA). All peptides were reconstituted in sterile distilled water containing 10% DMSO (Sigma Chemical Co.) and 1 mM DTT (Sigma Chemical Co.).

Cytotoxicity assay using vaccinia-infected target cells

B-LCL were infected with either recombinant vv-HCV vectors or a control vv at a multiplicity of infection of 5 to 10 plaque-forming units/cell, labeled with Na32CrO4 (DuPont NEN, Boston, MA), and incubated overnight at 37°C in 5% CO2. On the following morning, the B-LCL target cells were washed three times with cold R-10 media and incubated with effector cells at 37°C in 5% CO2 for 4 h. Cellular release of 32CrO4 into the supernatant was measured by the Top Count Microplate Scintillation Counter (Packard Instrument Company, Meriden, CT), and the percent specific cytotoxicity was calculated by the formula: percentage of lysis = ([experimental release – spontaneous release]/[maximum release – spontaneous release]) × 100%. Assays were excluded from analysis if the spontaneous release value was >30% of the maximum release value. Results are reported as the mean of triplicate values, with an SD of <5%. Samples were scored positive if the percentage of lysis at E:T = 100:1 was greater than 20% and at least 15% higher than the percentage of lysis for vv-Lac-infected B-LCL negative control.

Cytotoxicity assay using synthetic peptide-sensitized target cells

B-LCL were pelleted and resuspended in fresh R-20 and labeled overnight with Na32CrO4. Cells (1 × 106) were then resuspended in 0.2 ml of R-10, and peptide was added at concentrations ranging from 100 to 300 μg/ml, unless otherwise indicated. After a 1-h incubation at 37°C, cells were washed three times and used as targets in the cytotoxicity assay. For screening of large numbers of peptides in some assays, B-LCL were sensitized simultaneously with up to five overlapping peptides. Once the CTL epitope was localized to a single 20-mer peptide, fine mapping experiments were performed in which B-LCL targets were sensitized with various truncated peptides at concentrations ranging from 100 μg/ml to 10 pg/ml. The optimal epitope was defined as the shortest peptide truncation that resulted in greatest recognition of the B-LCL target, as determined by the lowest SD50 with a high lysismax arbitrarily defined as greater than 40% above background (see below).

HCV quantitation and serotyping

Viral load was measured by the HCV RNA 2.0 assay (Chiron Corp., Emeryville, CA), a branched DNA assay that has a lower limit of detection of 2 × 103 Eq/ml (26). HCV genotype was determined by the recombinant immunoblot assay HCV serotyping system (Chiron Corp.). This assay tests for serologic responses to five different serotype-specific peptides from the NS4 regions and two serotype-specific peptides from the core regions of the HCV genotypes for types 1, 2, and 3 (27).

Data analysis

HCV sequences were aligned and analyzed using Higgins Protocol on the MacDNASIS 3.5 software package (Hitachi Software, San Bruno, CA). CTL recognition of a peptide was assessed by peptide titration studies. The log dose-response relationship was modeled with the median-effect equation: percentage of lysis=lissmax=1/(1 + (SD50(peptide)*p)), in which lissmax is the expected percentage of lysis at saturating doses of peptide, SD50 is the sensitizing dose of peptide required to achieve one-half of lissmax, and m is the slope of the function (28, 29). The parameters for this nonlinear regression line were estimated by the Quasi-Newton procedure (30). Correlations between viral load and CTL responses were tested with the Mann-Whitney U test and the Spearman Rank Order Correlations test. All statistical analyses were performed using the Statistica for Windows 5.1 software package (StatSoft, Tulsa, OK).
as a negative control.

E2(NS1)NS2 in 5 subjects (Table I). No CTL clones were derived core/E1 in 1 subject, and clones to vv-E2(NS1)NS2 and/or vv-

assays, CTL clones were derived from 6 subjects: clones to vv-core/E1 in 2 subjects (91E and 94I), and vv-E2(NS1)NS2 and/or vv-E2(NS1)NS3 for the E2 envelope protein (Fig. 1). The latter two constructs also result in the expression of portions of the NS2 and NS3 proteins. These vectors allowed for the analysis of responses to the highly conserved core as well as the highly variable envelope proteins. Sufficient numbers of bulk expanded cells were available from 39 of the 44 subjects for screening of CTL responses with these vectors. Assays in which CTL activity was detected in a bulk culture are shown in Table I. An assay was considered positive if the percentage of lysis for a given vector was greater than 20% and at least 15% higher than the percentage of lysis for the vv-Lac control. The results demonstrate marked heterogeneity in the detectable intrahepatic CTL response among persons with chronic HCV hepatitis. Of the 39 subjects studied, 3 (8%) had detectable HCV-specific CTL activity in bulk cultures against B-LCL infected with vv-core/E1, and 6 (15%) had CTL activity against either vv-E2(NS1)NS2 and/or vv-E2(NS2/NS3. For each of these, CTL activity was confirmed subsequently by cloning (see below).

**Results**

**CTL activity from bulk cultures of liver-derived lymphocytes: recognition of endogenously processed structural proteins**

When these studies were initiated, only a limited number of vv vectors representing the entire HCV polyprotein subsequently became available. All infected individuals in our cohort were screened for CTL recognition of autologous B-LCL infected with the original vv vectors expressing the putative structural proteins of HCV-1. Three different recombinant vaccinia vectors were used: vv-core/E1 for the core and the E1 envelope proteins; and the vectors vv-E2(NS1)NS2 and/or vv-E2(NS2/NS3 for the E2 envelope protein (Fig. 1). The latter two constructs also result in the expression of portions of the NS2 and NS3 proteins. These vectors allowed for the analysis of responses to the highly conserved core as well as the highly variable envelope proteins. Sufficient numbers of bulk expanded cells were available from 39 of the 44 subjects for screening of CTL responses with these vectors. Assays in which CTL activity was detected in a bulk culture are shown in Table I. An assay was considered positive if the percentage of lysis for a given vector was greater than 20% and at least 15% higher than the percentage of lysis for the vv-Lac control. The results demonstrate marked heterogeneity in the detectable intrahepatic CTL response among persons with chronic HCV hepatitis. Of the 39 subjects studied, 3 (8%) had detectable HCV-specific CTL activity in bulk cultures against B-LCL infected with vv-core/E1, and 6 (15%) had CTL activity against either vv-E2(NS1)NS2 and/or vv-E2(NS2/NS3. For each of these, CTL activity was confirmed subsequently by cloning (see below).

**CTL activity of clones specific for structural proteins**

To confirm the presence of structural protein-specific CTL responses detected from bulk cultures and to further characterize these CTL responses, CTL cloning from the bulk expanded CD8 cells was performed in 37 subjects. Multiple clones (median, 100 clones; lower-upper quartiles, 84–132 clones) were established from each subject and tested for HCV-specific CTL activity. For each 8 subjects with positive bulk CTL assays, CTL clones with the same specificities were detected (Table I). Furthermore, additional CTL responses with different specificities were detected in three subjects: clones to vv-core/E1 in 2 subjects (91E and 94I), and clones to vv-E2(NS1)NS2 and/or vv-E2(NS1)NS2 in 1 subject (92N). Of the 24 subjects who did not have detectable CTL in bulk assays, CTL clones were derived from 6 subjects: clones to vv-core/E1 in 1 subject, and clones to vv-E2(NS1)NS2 and/or vv-E2(NS1)NS2 in 5 subjects (Table I). No CTL clones were derived from the 6 subjects in whom bulk assays had not been performed. These results indicate that screening multiple clones was more sensitive than bulk assays for detecting CTL and that the HCV-specific CTL were often below the magnitude required for detection in the bulk assay. For the 32 subjects in whom responses to the structural proteins were assessed by both bulk assays and CTL cloning, 14 (44%) had a detectable CTL response, with recognition of vv-core/E1 in 5 subjects (16%) and recognition of vv-E2(NS1)NS2 and/or vv-E2(NS2/NS3 in 12 (38%) subjects. These data indicate that CTL responses to the structural proteins, detected using this methodology, are present in the minority of persons with chronic HCV hepatitis, and suggest that responses to core are less frequent than to non-core Ags, despite the fact that the sequence of the core protein is more highly conserved among the different HCV strains (31).

**CTL recognition of the nonstructural proteins**

Nonstructural proteins also serve as targets for HCV-specific CTL (20), but the frequency of CTL responses to these proteins is less well defined. To further address this issue, vv expressing the nonstructural proteins of HCV-1 were used to assess CTL responses in a subset of subjects. Table II shows the results of screening for CTL responses using the expanded panel of vectors in subjects in which CTL responses to the nonstructural proteins were identified. Once again, bulk culture and cloning results were not always concordant, and the greatest sensitivity was obtained through a combination of both methods. CTL responses in bulk cultures and/or clones were observed to the NS4 protein in 3 of 9, NS5A protein in 2 of 9, and NS5B protein in 5 of 11 subjects tested.

A more comprehensive analysis of responses to the entire genome was performed on the last 22 subjects enrolled, due to the availability of additional vaccinia vectors. These studies included target cells expressing the complete translated polyprotein of HCV-H, a genotype 1a strain that is 96% homologous to HCV-1 at the peptide level as well as selected HCV-1 vectors. Of the 22 subjects screened with bulk assays and CTL cloning, CTL responses were detected in 10 (45%) (Table III). The percentage of cloned cells with HCV-specific activity ranged to as high as 42%. Two subjects (93P and 95I) were found to have CTL responses to the nonstructural proteins without detectable CTL responses to the structural proteins. These data indicate that the nonstructural proteins are important targets for HCV-specific CTL and that screening for CTL responses to the structural proteins alone will lead to an underestimation of the CTL response.
Subjects with negative bulk assays and detectable CTL clones and are presented here for comparative purposes. For subjects 91E and 91G (18); 92G, 92H, 92N (19); and 92T, 93I, 93K, and 94F (20), the epitope was the 11-amino acid peptide HDGAGKRVYYL, corresponding to aa 2794–2804 with an SD50 of 74 ng/ml and lysis max of 88% (Fig. 2C). This response was restricted by the HLA class I allele, B38 (Fig. 2D). Other optimal epitopes were defined in a similar fashion, with the exception of subjects 91E, 92G, and 92H, in which peptide titration studies were not performed and the minimum epitope was defined as the shortest peptide recognized. Some of these epitopes have been published previously (18–20) and are presented again to illustrate the scope of the CTL response to HCV (Fig. 3). Only one epitope (HLA-B7-restricted core epitope, GPRLGVRAT) was recognized by clones from more than one subject (92N and 93I). Otherwise, the CTL epitopes identified were heterogeneous (Table III). For example, the majority of the 17 individuals with HLA-A2, the most commonly expressed allele (Fig. 2), had a response to a single region of HCV, whereas 3 subjects had responses to 5 regions within HCV. For those subjects in which CTL responses to more than one epitope were identified, the magnitude of the response for each epitope, reflected by the relative number of clones with the different specificities, was also heterogeneous (Table III). For example, the majority of the CTL clones from subject 93I were directed against the structural protein, and one to the envelope, but the other one clone could not be maintained in culture for detailed characterization.

**Breadth of the CTL responses in chronic HCV infection**

From a total of 27 potentially different targeted regions of the virus in this cohort, 20 different cell lines could be maintained in long-term culture for complete characterization using synthetic HCV peptides to determine the optimal epitope recognized and the restricting HLA allele. For example, characterization of the NS5B-specific clone, 95I-C37, is shown in Figure 2. Using synthetic peptides (20 aa long, overlapping by 10 aa) spanning the NS5B protein, the epitope was initially determined to be located within aa 2791–2810 (Fig. 2, A and B). Peptide truncation studies using truncations within aa 2791–2810 show that the optimal epitope was the 11-amino acid peptide HDGAGKRVYYL, corresponding to aa 2794–2804 with an SD50 of 74 ng/ml and lysis max of 88% (Fig. 2C). This response was restricted by the HLA class I allele, B38 (Fig. 2D). Other optimal epitopes were defined in a similar fashion, with the exception of subjects 91E, 92G, and 92H, in which peptide titration studies were not performed and the minimum epitope was defined as the shortest peptide recognized. Some of these epitopes have been published previously (18–20) and are presented again to illustrate the scope of the CTL response to HCV (Fig. 3). Only one epitope (HLA-B7-restricted core epitope, GPRLGVRAT) was recognized by clones from more than one subject (92N and 93I). Otherwise, the CTL epitopes identified were distributed throughout the genome. Of the five CTL responses to the core/E1 construct, three were mapped to the core protein, and one to the envelope, but the other one clone could not be maintained in culture for detailed characterization.

The character of the CTL response in each subject was heterogeneous. Of the 16 subjects with a detectable CTL response, 10 had a response to a single region of HCV, whereas 3 subjects had responses to 2 regions, 2 subjects had responses to 3 regions, and 1 subject had responses to 5 regions within HCV. For those subjects in which CTL responses to more than one epitope were identified, the magnitude of the response for each epitope, reflected by the relative number of clones with the different specificities, was also heterogeneous (Table III). For example, the majority of the CTL clones from subject 93I were directed against the structural proteins, whereas those from subject 94F were directed against the nonstructural proteins.

The distribution of the number of subjects who expressed each HLA-A and HLA-B class I allele is shown in Figure 4, along with the number of subjects demonstrating CTL responses restricted by each HLA allele. The distribution of HLA alleles in this cohort is comparable with previously published data for North American populations (32). There was no evidence of a predominant HLA class I-restricting element in the 20 identified CTL epitopes. Of the 17 individuals with HLA-A2, the most commonly expressed allele

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**Table I. Lysis of target cells expressing HCV structural proteins by liver-infiltrating lymphocytes from persons with chronic HCV hepatitis**

<table>
<thead>
<tr>
<th>No.</th>
<th>ID</th>
<th>Effector E:T</th>
<th>v-v-Core/E1 % Lysis</th>
<th>vv-E2(NS1)/NS2 or v-v-E2(NS2)/NS3 % Lysis</th>
<th>vv-Lac % Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>91E</td>
<td>Bulk</td>
<td>100:1, 24, 54%</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>92G</td>
<td>Bulk</td>
<td>100:1, 24, 7%</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>92H</td>
<td>Bulk</td>
<td>100:1, 8,</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>92N</td>
<td>Bulk</td>
<td>100:1, 8</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>92T</td>
<td>Bulk</td>
<td>100:1, 21</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>6</td>
<td>93I</td>
<td>Bulk</td>
<td>100:1, 37</td>
<td></td>
<td>41</td>
</tr>
<tr>
<td>7</td>
<td>94I</td>
<td>Bulk</td>
<td>100:1, 20</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>94K</td>
<td>Bulk</td>
<td>100:1, 22</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>91G</td>
<td>Bulk</td>
<td>100:1, 6</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>93C</td>
<td>Bulk</td>
<td>100:1, 11</td>
<td></td>
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<tr>
<td>11</td>
<td>93K</td>
<td>Bulk</td>
<td>100:1, 2</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>94C</td>
<td>Bulk</td>
<td>100:1, 13</td>
<td></td>
<td>26</td>
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<tr>
<td>13</td>
<td>94F</td>
<td>Bulk</td>
<td>100:1, 11</td>
<td></td>
<td>5</td>
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<tr>
<td>14</td>
<td>95K</td>
<td>Bulk</td>
<td>100:1, 10</td>
<td></td>
<td>7</td>
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<tr>
<td></td>
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</table>

**Table II. Lysis of target cells expressing HCV nonstructural proteins by liver-infiltrating lymphocytes from persons with chronic HCV hepatitis**

<table>
<thead>
<tr>
<th>No.</th>
<th>ID</th>
<th>Effector E:T</th>
<th>vv-NS4 % Lysis</th>
<th>vv-NS5A % Lysis</th>
<th>vv-NS5B % Lysis</th>
<th>vv-Lac % Lysis</th>
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<tr>
<td>1</td>
<td>93I</td>
<td>93I-C16</td>
<td>10:1, 5</td>
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<td></td>
<td>75</td>
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<tr>
<td>2</td>
<td>94F</td>
<td>94F-C88</td>
<td>10:1, 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>94I</td>
<td>94I-C69</td>
<td>10:1, 4</td>
<td></td>
<td></td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>94L</td>
<td>94L-C125</td>
<td>10:1, 22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>94K</td>
<td>94K-C10</td>
<td>10:1, 2</td>
<td></td>
<td></td>
<td>58</td>
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<tr>
<td>6</td>
<td>95I</td>
<td>95I-C3</td>
<td>10:1, 9</td>
<td></td>
<td></td>
<td>43</td>
</tr>
</tbody>
</table>

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* HCV-specific cytolytic activity of bulk cultures and limiting dilution clones were tested for recognition of HCV structural proteins in a standard 4-h 51Cr release assay. Results are shown only for the 5 persons with positive responses of the 11 subjects tested (the bulk culture of subject 93I was not tested for CTL activity to the nonstructural proteins). Recombinant vaccinia (vv-HCV) vectors (see Fig. 1) were used to transiently express HCV structural proteins on autologous B-LCL target cells (except subject 94F, where HLA-matched B-LCL were used). vv-Lac = negative control. Effector cells were either bulk (uncloned) cultures or specific limiting dilution clones derived from the bulk cultures. Values represent the mean percent of lysis of triplicate values for the bulk assays and duplicate values for clone assays. Assays are scored as positive (in bold) if the percent of specific lysis was >20% and at least 15% higher than the percent of lysis of vv-Lac negative control. neg = negative on previous assay but not repeated.
in our study population, 7 had detectable CTL responses, but only 1 individual had an HLA-A2-restricted response. Similarly, 5 of 11 individuals who expressed HLA-B7 had detectable CTL responses, but only 2 individuals had detectable CTL responses to the well-conserved HLA-B7-restricted epitope in the core protein. Taken together, these data indicate that the CTL response targeted multiple regions, and there was no evidence for an immunodominant epitope, either on a protein or HLA level, among this HLA-diverse population.

**CTL response and viral load**

We also examined the potential relationship between viral load and presence or absence of detectable CTL responses. CTL responses were detected in persons infected with HCV serotypes 1, 2, and 3 strains, and were detected over a wide range of viral load. In an analysis restricted to the subgroup of 22 subjects who were screened for CTL responses to the entire genome of HCV using both bulk assays and CTL cloning, which is the most sensitive method for detecting CTL responses in our hands, the range in viral load was similar in those with and without detectable CTL responses (Fig. 5). Hence, there was no statistically significant correlation between viral load and presence of detectable CTL (p = 0.32). When the analysis was expanded to include 32 subjects who were screened for CTL responses to the structural proteins using bulk assays and CTL cloning, there was still no correlation between CTL responses and viral load (data not shown, n = 32, p = 0.53). However, analysis of the relationship between viral load and CTL would ideally include assessment of type-specific responses to autologous virus, quantitation of CTL precursors, and multiple biopsies to ensure that sampling bias is not introduced, but technical limitations precluded such studies.

**Influence of HCV genotype on the CTL response**

CTL responses may have been present in vivo, but not detected because of sequence differences between the infecting strain and the vv-HCV constructs used in our study. The majority of persons with CTL responses to HCV-1 Ags had corresponding CTL responses to HCV-H Ags, with the exception of subjects 92N and 93I, in which CTL recognized epitopes that corresponded to HCV-1, but not HCV-H sequence (data not shown). The effect of natural sequence variation was examined by comparing CTL recognition of peptide sequences corresponding to different published strains at a previously identified epitope. An example is shown in Figure 6 for an epitope in the highly variable E2 envelope protein. Clone 94F-C35 recognized peptides corresponding to the type 1a strain, HCV-1, as well as to the type 1b strain, HCV-BK (33), but not to two other 1b strains, HCV-J (34) and HCV-JK (35), nor the 2a strain, HCV-J6 (36).

To assess the degree of homology between the different strains, the published peptide sequence of HCV-1 (23) was aligned and compared with the sequences of another 1a strain, HCV-H (24), the 1b strain HCV-J (34), the 2a strain HC-J6 (36), and the 3a strain HCV-K3A (37). At the peptide level, the core protein is very well conserved, as there is significant homology with strains from the same genotype: HCV-H (98.4%), HCV-J (97.3%), and, to a lesser degree, in strains of different genotypes: HC-J6 (87.4%) and HCV-K3A (85.8%). There are significant differences in the envelope proteins, but the nonstructural proteins are relatively similar, with homologies on the order of 96% within the same subtype, 85% within the same genotype, and 67% between different genotypes. Although the serotyping assay could not distinguish between subtypes 1a and 1b, at least 31 (70%) were infected with serotype 1 strains, indicating that the vectors used (1a strains) should be highly homologous to the infecting HCV strain in the core protein and, to a lesser degree, the nonstructural proteins. Four of the sixteen subjects with detectable CTL responses were infected with non-serotype 1 strains (one serotype 2 and three serotype 3, Table III). Subject 94F (serotype 3) was found to have CTL responses to five different epitopes, subject 94I (serotype 2) had CTL responses to three different epitopes, whereas subjects 92G (serotype 3) and 95K (serotype 3) both had CTL responses to a single epitope in the envelope protein. These results indicate that CTL cross-reactivity among widely divergent HCV strains does occur, but minor sequence variation can lead to less efficient or complete loss of recognition by HCV-specific CTL, which is similar to what has been observed for other viruses (38–40).
Discussion

We have conducted a cross-sectional study of the intrahepatic CTL response in a cohort in which chronic HCV infection had been established. In this cohort, the CTL response was found to be quite heterogeneous. The breadth of response ranged from one to five different epitopes recognized. CTL responses were directed at regions scattered throughout the translated polyprotein, with no evidence for an immunodominant response to a particular protein. Of those persons in whom recognition of HCV proteins in both bulk expanded and cloned CTL was tested, 45% had detectable responses. Responses to the core protein, which is 97% homologous among 1a and 1b strains and 86% homologous among non-genotype 1 strains, were detected less frequently. These findings are similar to another study by Nelson et al. in which only bulk expanded cells were assessed (12). Both studies indicate that the core protein is targeted in a minority of chronically infected persons, and our data indicate that lack of detectable responses in some persons persists even when more sensitive assays, including CTL cloning, are performed. However, it should be noted that although the data indicate that the intrahepatic CTL are HCV specific, these experiments cannot distinguish whether they were in an activated state in vivo, whether they were nonactive memory cells, or a combination of the two.

The majority of persons in this cohort did not have a detectable CTL response, despite ongoing HCV replication. It is possible that CTL were not present in the liver of these individuals because of impaired CTL induction in vivo, or clonal anergy/exhaustion/deletion (41, 42). Alternatively, CTL may have been present, but not detected, due to sampling or technical limitations in the detection strategies used. CTL responses to the nonstructural proteins may have been present, but not detected in the subjects who were only screened for responses to the HCV structural proteins. Subject 95I-C37 is an example of an individual who had a CTL response only to NS5B, and therefore would have scored as a CTL nonresponder if only tested with vectors expressing the structural proteins. However, the 45% detection rate of CTL in the subjects who were screened for CTL responses to the entire HCV-H genome was not different from the rate of 44% in those who were only screened for CTL responses to the structural proteins. Furthermore, CTL responses could be missed if the CTL recognized autologous viral sequences, but did not cross-react with either HCV-1 or HCV-H, our reference sequences. For example, 94F-C35 is a clone that can...
tolerate some changes within the epitope, but lost CTL recognition with further aa substitutions.

Another possible explanation for the infrequent detection rate is that HCV-specific CTL may have been present in the bulk cultures, but at such a low frequency they could not be detected. Several HCV-specific CTL clones were derived from bulk cultures in which the CTL assay was initially negative. Detection might be improved by using Ag stimulation to preferentially stimulate the subset of cells within the bulk culture that had specificity for HCV. Other studies have used peptides from well-conserved regions of HCV selected on the basis of their predicted binding affinity to common HLA class I alleles (43–45). Indeed, several groups have successfully used this strategy for detecting CTL from PBMC, in which the frequency of HCV-specific CTL is felt to be extremely low (14, 15, 46, 47). However, such strategies are complicated by the fact that they can potentially induce CTL in uninfected persons (48–50). In addition, type-specific responses might be missed by peptide stimulation strategies as well, in that the predicted peptide sequence may not be present as part of the infecting viral sequence. Furthermore, the predicted motifs are currently only available for selected HLA class I alleles. Our data suggest that even in individuals with common HLA class I alleles, such as A2 and B7, CTL responses may be present in these individuals, but restricted by alleles that are encountered less frequently. For example, subject 94I expresses HLA-A2, A31, B7, B60. After screening for CTL responses to the entire HCV genome, the two CTL responses detected were restricted by HLA-A31 and B60.

The studies presented in this work add to the number of epitopes identified to be targeted by CTL in HCV infection (13–15, 18–20, 46, 47, 51–55) and begin to establish the breadth and specificity of responses in an HLA-diverse population. Given the limitations for detecting CTL responses, this can still be an underestimate of the breadth of the CTL response. When present, there was evidence of a dominant response within each of the individuals with detectable CTL responses, as evidenced by the different frequencies of clones detected to different epitopes within individuals who had CTL responses to more than one epitope (Table III), and there was no evidence for an immunodominant response that was common to different individuals. Only one epitope was recognized by more than one individual, consistent with what we had observed in an earlier analysis of a smaller cohort (20). However, in these expanded studies, this HLA-B7-restricted epitope in the core protein was only found in 2 of the 11 subjects known to be HLA-B7 positive. Indeed, three subjects (92G, 94F, and 94I) who were also HLA-B7 positive had detectable CTL responses to other epitopes, but not the B7-restricted epitope in the core protein. The lack of a detectable CTL response to the core protein cannot be explained by subtle differences in the HLA-B7 molecule between these individuals, since CTL from subject 93I also recognized this epitope presented on B-LCL from subjects 94F and 94I (data not shown).
Taken together, the data indicate that there is marked variability in the breadth and specificity of CTL responses to HCV in chronically infected persons. It is not known how HCV is able to persist in the face of this potential immune pressure. Although some studies have found a statistically significant association between the presence of detectable CTL and viral load, the magnitude of the difference in median viral load is modest at less than 1 log (12, 13). We have not found a difference in viral load between those with and without detectable HCV-specific CTL responses, despite using a detailed and sensitive analysis that included CTL cloning. To date, studies of HCV-specific CTL have been performed in chronically infected individuals and have not been designed to assess the potential role of CTL in viral clearance. Such studies are not easy to perform, since it is difficult to identify acutely infected individuals in whom immune mediated clearance of HCV might occur such that CTL responses can be measured longitudinally. In a recent report of an individual with acute HIV-1 infection by Borrow et al., a very vigorous CTL response (precursor frequency about 1/17 PBMC) to the envelope protein gp160 was associated with mutations within the CTL epitope and immune escape (56). CTL escape during experimental primary HCV infection has also been noted in the chimpanzee model (57). Issues including what constitutes a sufficient HCV-specific CTL response, either in terms of the magnitude 

![FIGURE 4](image4.jpg)

**FIGURE 4.** Frequency of CTL response restricted by each HLA class I allele. The distribution of the total number of subjects (open bars) with each HLA-A allele (5A) or HLA-B allele (5B), and the number of subjects with HCV-specific CTL restricted by that particular HLA allele (filled bars) are shown.

![FIGURE 5](image5.jpg)

**FIGURE 5.** Lack of correlation between viral load and magnitude of the CTL response. The range in viral load is shown for the 22 subjects who were screened for CTL responses against the entire genome using bulk assays and CTL cloning. No significant difference in viral load was noted between those with and those without detectable CTL responses. Viral load was measured by the HCV RNA 2.0 assay, which had a lower limit of detection of $2 \times 10^5$ Eq/ml (dotted line).
or the breadth of response, the mechanisms of HCV persistence in the face of a CTL response, and whether augmentation of CTL responses might provide a therapeutic benefit, remain to be clarified. In addition, the relationship between CTL and other aspects of HCV-specific immunity, including proliferative responses (58–60) and neutralizing Abs (61–63), needs further definition.

In summary, our results indicate that the CTL response to HCV is quite heterogeneous in an HLA-diverse population of chronically infected individuals, and that multiple epitopes are targeted, with no evidence of an immunodominant CTL epitope. It is clear from this and other studies (12–15) that CTL, in and of themselves, are insufficient for viral clearance once chronic infection has been established. Although we did not find a correlation between the presence of a detectable CTL response and viral load, rigorously designed experiments that allow for the assessment of CTL responses to all potential Ags from the entire translated polypeptide of the autologous viral sequences will be needed to fully address this question.

References


FIGURE 6. Peptide titration assay to determine the tolerance of CTL for variations in the peptide epitope. To assess the tolerance of CTL for sequence variation, various peptides corresponding to the sequences of different published strains of HCV at an HLA-A11-restricted epitope in the E2 envelope protein were synthesized. These peptides were incubated with an HLA-A11-expressing B-LCL target cell line at various peptide concentrations ranging from 50 μg/ml to 1 ng/ml. Clone 94F-C5 recognized the type 1a sequence (HCV-1: TINITYIFK, filled squares) optimally with an SD50 of 19 ng/ml. The clone also recognized the 1b sequence with a single aa substitution (HCV-BK, filled triangles) at a higher SD50 of 97 ng/ml, but it failed to recognize additional changes present in the other two 1b sequences (HCV-JK1, open squares; HCV-J, open triangles) or the 2a sequence (HCV-J5, open circles). Where CTL recognition was present, solid lines represent the regression line (R > 0.99), as described previously.


